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17- β -estradiol in relation to age at menarche and adult obesity in premenopausal women

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Background: We hypothesize that premenopausal endogenous estradiol may be associated with age at menarche and adult overweight and obesity, potentially contributing to breast cancer risk.

Methods: We assessed age at menarche by questionnaire among 204 healthy Norwegian women, aged 25–35 years. Measures of body composition included body mass index (BMI kg/m²), waist circumference (WC cm), waist to hip ratio (WHR) and fat percentage (DEXA). Daily salivary 17 β -estradiol (E₂) concentrations were collected throughout one entire menstrual cycle and assessed by radioimmunoassay (RIA). Linear regression analyses and linear mixed models for repeated measures were used and potential confounding factors and effect modifiers were tested.

Results: Among women with an early age at menarche (≤ 12 years), the overall mean salivary E₂ concentration increased by 3.7 pmol/L (95% confidence interval, 1.8-5.7) with each 9.8 cm (1 SD) increase in WC, which represents a 20.7 % change in the mean for the total group. Among the same early maturers, a 1 SD (0.06) change in WHR was directly associated with a 24.0 % change in mean E₂ concentration for the total group.

Conclusion: Our findings support the hypothesis that early age at menarche, together with adult overweight and obesity, result in high levels of 17- β -estradiol throughout the menstrual cycle. However, we have measurements only from one menstrual cycle.

Introduction

The increasing breast cancer incidence worldwide follows trends toward earlier age at menarche (Onland-Moret *et al.*, 2005), higher rates of obesity (IARC Handbook of Cancer Prevention, 2002;Ballard-Barbash *et al.*, 2006) and unfavourable metabolic profiles (visceral obesity, glucose intolerance, hypertension and dyslipidemia). Age at menarche (Key *et al.*, 2001;Clavel-Chapelon and Gerber, 2002;Butler *et al.*, 2000;Kelsey *et al.*, 1993) and obesity (IARC Handbook of Cancer Prevention, 2002;Ballard-Barbash *et al.*, 2006) have been identified as risk factors for breast cancer. For each 1-year delay in menarche, the risk of breast cancer decreases by around 5%, and this menarcheal effect may be stronger in premenopausal than in postmenopausal women (Key *et al.*, 2001), indicating a time-dependent effect. Excessive body weight is associated with an increased risk of postmenopausal breast cancer, but has most often been observed to decrease the risk of premenopausal breast cancer (IARC Handbook of Cancer Prevention, 2002).

A younger age at menarche is associated with higher cumulative exposure to ovarian hormones which may play a critical role in breast carcinogenesis (Pike *et al.*, 1993;Bernstein, 2002). Estrogens are considered to stimulate ductal growth and cell proliferation of breast epithelial cells. High levels of serum estrogens are associated with an increased risk of breast cancer among postmenopausal women (Key *et al.*, 2002), but the role of estrogens in the etiology of premenopausal breast cancer are more complex (Sturgeon *et al.*, 2004;Eliassen *et al.*, 2006;Key *et al.*, 2002) and is more difficult to study because of the complexity of measuring the cyclic variation of estrogens (Bernstein, 2002;Kaaks *et al.*, 2005).

Adiposity appears to be both a cause and a consequence of early age at menarche (Brown *et al.*, 1996). Studies indicate that factors influencing energy balance and metabolism, such as physical activity and body composition, are strong predictors of age at menarche (Kirchengast *et al.*, 1998;Chavarro *et al.*, 2005). The improvement in living conditions,

nutrition and increase in energy intake, combined with a reduction in energy expenditure, may explain why age at menarche has dropped in Norway from about 16 years for women born around 1830, to 13 years for those born around 1960 (Rosenberg, 1991) and 1985 (Lien *et al.*, 2006). Importantly, several studies indicate that early maturation seems to increase overall adult fatness (Kirchengast *et al.*, 1998; Key *et al.*, 2001).

Little is known about the association between adult levels of estradiol throughout an entire menstrual cycle and age at menarche and adult body composition. We have previously observed that an unfavorable metabolic profile, including being overweight (Furberg *et al.*, 2005) and physically inactive (Jasienska *et al.*, 2006), is associated with increased levels of free E₂ throughout a menstrual cycle. In addition, we have previously observed that unfavorable metabolic profile reflected by high energy intake and weight gain, together with physical inactivity, increases the risk of postmenopausal breast cancer (Thune *et al.*, 1997; Jasienska and Thune, 2001; Thune *et al.*, 1998; Furberg *et al.*, 2004).

Thus, the aims of the present study were to elucidate the association between age at menarche and premenopausal 17- β -estradiol levels throughout a menstrual cycle, and whether an unfavorable energy balance as expressed through a higher fat mass in adulthood might influence such an association.

Materials and Methods

Participants and study design

A total of 204 women, aged 25–35 years were invited to participate in the Norwegian EBBA study during 2000–2002. Women who met the following criteria were included: self-reported regular menstruation (normal cycle length: 22–38 days within the previous 3 months), no use of steroid contraceptives and no pregnancy or lactation over the previous 6 months, no history of gynecological disorder and no chronic disorders (e.g. diabetes, hypo-/hyperthyroidism).

Participants were subsequently enrolled into the study and came to the Department of Clinical Research, University Hospital North Norway (UNN), at a scheduled time (Furberg *et al.*, 2005).

All the participating women signed an informed consent form. The study was approved by the Regional Committee for Medical Research Ethics and the Norwegian Data Inspectorate.

Questionnaires

We used questionnaires (self and interviewer-administered by trained personal) to collect information on age at menarche, marital status, education, ethnicity, reproductive history, lifetime total physical activity, previous use of hormonal contraceptives, family history of cancer, smoking [“current smoker” (yes/ no), “how many cigarettes per day”] and alcohol [“do you drink alcohol” (yes/ no), “units of alcohol”]. In this questionnaire, all of the participants in particular answered questions about age at menarche at home, and then they went through the questions administrated by trained personal. Recall and a memory-probing aid that included a lifetime calendar were used.

The validity of the response to the questions on smoking and alcohol has previously been studied (same age group, both men and women) by measurements of serum thiocyanate concentrations (Knutsen and Knutsen, 1991) and levels of serum γ -glutamyltransferans levels (Nilssen and Forde, 1991) respectively. We collected dietary data on seven different days during the menstrual cycle (days 3–6 and 21–23) by a pre-coded food diary (Lillegaard *et al.*, 2005).

Clinical parameters

Study participants met fasting on three subsequent visits during the collection period: first visit (days 1–5 of the menstrual cycle), second visit (days 7–12) and third visit (days 22–25) at the Department of Clinical Research, UNN, Tromsø. For the first visit, the participants met on the first day possible after onset of menstrual bleeding. Anthropometric measurements were taken with participants wearing light clothing and no footwear: height was measured to the nearest half-centimeter and weight to the nearest 0.1 kilogram on an electronic scale. Body mass index (BMI) was used to estimate relative weight. Waist circumference (WC) was measured in a horizontal line 2.5 cm above the umbilicus; hip circumference was measured at the largest circumference, both measured to the nearest 0.5 cm. Waist to hip ratio (WHR) was estimated. The participants underwent whole-body scan (days 7-12) using dual energy X-ray absorptiometry (DEXA – DPX-L 2288, Lunar Radiation Corporation, Madison, WI, USA), and the percentages of total and truncal fat tissue were estimated using standard Lunar software. In this study we have presented analysis using total tissue fat. Using truncal fat gave similar results.

Serum samples

Fasting serum blood samples were drawn from an antecubital vein in the morning on each of the three visits during the menstrual cycle. The blood was centrifuged and the serum separated. Serum concentrations of 17- β -estradiol were measured in fresh sera at the Department of Clinical Chemistry, UNN (Furberg *et al.*, 2005).

Hormone analysis

Women collected samples of their own saliva to plastic tubes pretreated with sodium azide at

home once a day, preferentially in the morning, for one entire menstrual cycle (Furberg *et al.*, 2005). They started on the first day of bleeding, according to previously established collection protocols developed at the Reproductive Ecology Laboratory at Harvard University, USA (Lipson and Ellison, 1996).

17- β -estradiol concentrations were measured in daily saliva samples using an ^{125}I -based radioimmunoassay (RIA) kit (#39100, Diagnostic Systems Laboratory, Webster, TX, USA), along with published modifications to the manufacturer's protocol (Furberg *et al.*, 2005). All samples were run in duplicate. All of a woman's samples were run in the same batch, with women randomly assigned to batches. CV's were calculated from high to low value pools (appropriate to the range of each steroid) that were run with each batch (Furberg *et al.*, 2005).

The sensitivity of the E₂ assay (the lowest concentration of E₂ distinguishable from 0 at the 95% level) was 4 pmol/L. Average intra-assay variability (estimated from the 50% binding point of the standard curve) was 9%, and the inter-assay variability ranged from 23% for lower values (15 pmol/L) to 13% for higher values (50 pmol/L). Salivary assays do have higher variability than serum assays because they are measuring levels that are one to two orders of magnitude lower in concentration. This may impact the results so the lower values (in the tail of the cycle) will have greater variability.

Before statistical analysis, all cycles were aligned to the day of ovulation following published methods (Lipson and Ellison, 1996), based on the identification of the E₂ drop at the mid-cycle (day 0), which provides a reasonable estimate of the day of ovulation. The values for 20 consecutive days for E₂ (day -10 to + 9) from each cycle, aligned on day 0, were used in data analyses. Satisfactory identification of the mid-cycle E₂ drop could not be made for 14 women and their cycles were not aligned. In detail to study the total variation in estradiol concentration throughout a menstrual cycle and as anovulatory cycles are associated

with low estradiol exposure, all cycles, both anovulatory and ovulatory cycles, were included in this study.

Overall mean salivary E₂ concentration was calculated for all 204 women, while additional indices (i.e. follicular value) were calculated for the 190 women with aligned cycles. A mean follicular value was calculated by averaging E₂ values for days -7 to -1. A mean luteal value was calculated by averaging the E₂ values for cycle days 0 to + 11.

Statistical analysis

The study population was divided into three subgroups of age at menarche: ≤ 12 years, 12–14 years and ≥ 14 years, based on the variation in age at menarche in a general western population. The various groups of age at menarche were then compared with regard to selected characteristics of the study population. We used one-way analysis of variance for continuous variables and χ^2 tests for categorical variables.

Age- and multivariate adjusted linear regression analyses were used to study the associations between mean salivary E₂ concentration (overall, follicular and luteal phases of the menstrual cycle), age at menarche and different measures of body composition. Possible interactions were studied.

In order to study whether variation in adult excess weight and fat distribution modified the association between age at menarche and salivary E₂ concentrations, the body composition variables were dichotomized at the 75th percentile (75 percentile BMI: ≥ 26.5 kg/m², 75 percentile DEXA total fat: 40 %, 75 percentile WC: ≥ 84 cm and 75 percentile WHR: ≥ 0.80), and age at menarche was categorized as ≤ 12 years and > 12 years. Additionally, the groups of women in these upper quartiles of BMI, DEXA total fat, WC and WHR were defined as being overweight, and/or obese (NIH and WHO definition; overweight: BMI ≥ 25.0 kg/m², obese: BMI ≥ 30.0 kg/m²).

We used a linear mixed model for repeated measures to study salivary E_2 concentrations throughout the entire menstrual cycle in relation to age at menarche and body composition. Different co-variance structures were explored and the results using heterogeneous Toeplitz are presented. Dunnett's method was used for multiple comparisons.

Age adjusted and multivariate adjusted results are presented. Based on biological plausibility and common knowledge possible covariates such as age, birth weight, energy intake, smoking, alcohol, previous use of hormonal contraceptives, time to establishment of regular cycles, age at first birth, number of children and physical activity were tested in the model. The following variables contributed and were included in the final model; age, birth weight, smoking, physical activity. As none of the multivariate analyses gave different results compared to age adjusted analyses, we present figure 1 only with age adjusted results.

Collinearity was of no concern as Pearson's correlation coefficient was ≤ 0.2 for all pairs of independent variables in the models. E_2 measurements at the beginning and the end of the cycles are encumbered with higher coefficient of variation; therefore we included E_2 measurements from cycle day -10 to +9. SAS statistical package version 9.1. was used.

Results

The participating 204 healthy women were, on average, aged 30.7 years (24.9-35.9 years) and reported a mean age at menarche of 13.1 years (9.2-19.5 years). The mean BMI was 24.4 kg/m^2 ($16.9\text{-}39.8 \text{ kg/m}^2$), WC 79.5 cm (61.0-116.0 cm) and percentage fat 34.2% (16.7-52.1%) (Table II). Mean salivary E_2 concentration was 17.9 pmol/L (not shown in table). Women with an age at menarche below 12 years were shorter ($p = 0.03$) and tended to have a higher BMI, a larger WC and a higher fat percentage than women with later age at menarche ($>12\text{-}14$ and ≥ 14 years) (Table I).

When studying estimated changes in overall mean salivary E₂ concentrations for the total group, by changes (1 SD) in different body composition measures, we observed that an increase in any of these measures of body composition resulted in a multivariate adjusted increase in E₂ concentration (Table II). For each 3.8 kg/m² (1 SD) increase in BMI, the overall mean age-adjusted level of E₂ increased by 2.2 pmol/L (95% confidence interval, CI, 1.0-3.4), which equals a 12.3% increase in mean overall concentration of E₂ for the total group. For each 9.8 cm (1 SD) increase in WC, the overall mean concentration of E₂ increased by 1.6 pmol/L (95% CI, 0.4-2.8), which equals an 8.9% increase in mean overall concentration of E₂ for the total group. Performing the same analyses but excluding the 14 women who did not have a mid-cycle E₂ drop, gave smaller estimated changes in salivary E₂, but the conclusion did not change, 1 SD increase in the different body composition variables gave significant increase in E₂ (data not shown).

We observed no difference in estimated changes in E₂ by different measures of body composition between the follicular and luteal phases of the menstrual cycle (Table II).

In order to elucidate whether age at menarche influenced our results, stratified analyses by subgroups of age at menarche was performed (Table III). Women with early age at menarche (≤ 12 years) had an increase in overall mean E₂ concentration by 3.7 pmol/L (95% CI, 1.8-5.7) by 9.8 cm (1SD) increase in WC ($p_{\text{interaction}}=0.07$); with each 0.06 (1 SD) increase in WHR, the overall mean E₂ concentration increased by 4.3 pmol/L (95% CI, 2.0-6.5) ($p_{\text{interaction}}=0.03$) and with each 7.6% (1SD) increase in total fat percentage, the overall mean E₂ concentration increased by 2.9 pmol/L (95% CI, 0.2-5.5) ($p_{\text{interaction}}=0.22$). These results equal a change in mean overall concentration of E₂ for the total group of 20.6%, 24.0% and 16.2%, respectively. Among women with age at menarche >12 -14 years and ≥ 14 years, no significant associations were seen with adult body composition.

To examine whether parity influenced our result, we performed analysis stratified by parity. Among women with early age at menarche (≤ 12 years), the relationship between measures of body fatness and overall E_2 concentration was strongest in the subgroup of non-parous women (data not shown). With each 0.06 (1 SD) increase in WHR, overall mean E_2 concentration increased by 6.1 pmol/L (95% CI, 1.5-10.9). However, no interaction between age at menarche, parity and the different anthropometric measurements were found and there were small numbers in each group.

We examined the mean E_2 concentrations by cycle day throughout the whole menstrual cycle in four groups stratified by age at menarche ≤ 12 years and >12 years and body fat and distribution measures at the 75% percentile (Fig. 1). Women characterized by both early age at menarche (≤ 12 years) and excess WC (≥ 84 cm, 75th percentile) or WHR (≥ 0.80 , 75th percentile) had age adjusted mean salivary E_2 profiles that were higher throughout the cycle, than the rest of the study population (Fig. 1).

Using a linear mixed model for repeated measures, we examined how the mean E_2 concentration by cycle day in women with both early age at menarche and excess body weight (groups II, Fig. 1) differed from the concentrations in the other groups of women described. Figure 1 is presented with age adjusted results, as none of the multivariate analyses gave different results compared to age adjusted analyses.

The difference between women with age at menarche ≤ 12 and $BMI \geq 26.6$ kg/m² (group II, Fig. 1a) and the other groups of women described in Fig. 1a was significant for group I ($p=0.04$) and III ($p=0.04$), but not for group IV ($p=0.43$). There was no statistically significant interaction between the dichotomized menarche variable and the dichotomized variable of BMI ($p_{\text{interaction}}=0.8$).

There were no significant differences between women with age at menarche ≤ 12 and DEXA % total fat $\geq 40\%$ (group II, Fig. 1b) and the other groups of women described in Fig.

1b (groups II and I, $p=0.17$; groups II and III, $p=0.15$; groups II and IV, $p=0.63$). There was no statistically significant interaction between the dichotomized menarche variable and the dichotomized variable of DEXA % total fat ($p_{\text{interaction}}=1.0$).

We examined how the mean E_2 levels by cycle day throughout the whole cycle in women with both age at menarche ≤ 12 and $WC \geq 84$ cm (group II, Fig. 1c) differed from the levels in each of the other groups of women described in Fig. 1c and observed significant differences for all comparisons (groups II and I, $p=0.004$; groups II and III, $p=0.03$; groups II and IV, $p=0.01$). There was a statistically significant interaction between the dichotomized menarche variable and the dichotomized variable of WC ($p_{\text{interaction}}=0.02$).

Finally, the same pattern was observed in women with age at menarche ≤ 12 and $WHR \geq 0.8$ (group II, Fig. 1d) and the other groups of women described in Fig. 1d (differences between group II in Fig. 1d and the other; group I, $p=0.003$; group III, $p=0.04$; group IV, $p=0.007$). There was a statistically significant interaction between the dichotomized menarche variable and the dichotomized variable of WHR ($p_{\text{interaction}} < 0.001$).

Discussion

To our knowledge this is the first study to support the hypothesis that early age at menarche in combination with adult overweight and obesity, might result in an overall increase in concentrations of free, biologically active E_2 . We observed a clear pattern after adjustments for potential confounders of a 16-24% increase in E_2 concentration among women reporting early age at menarche in combination with adult overweight and obesity.

Several studies have suggested that lifestyle related factors, including metabolic profile, and reproductive factors as age at menarche, separately affect breast cancer risk by altering exposure to estrogens (Verkasalo *et al.*, 2001; Jasienska and Thune, 2001; Bernstein, 2002). Women with early age at menarche have been observed to have higher levels of serum

E₂ throughout a cycle (Apter and Vihko, 1985;Apter and Vihko, 1983) and higher levels of urinary estrogen metabolites (Windham *et al.*, 2002) than women with later age at menarche. Evidence is becoming more consistent that there is an inverse association between physical activity and estrogens (Irwin, 2006;Williams, 2003).

Additionally, several studies have observed an association between early age at menarche and adult obesity (Laitinen *et al.*, 2001;Kirchengast *et al.*, 1998). It has been recognized that body fat is important for the initiation and maintenance of reproductive function in women, explaining why weight gain accelerates and energy restriction delays the onset of menses (Adair and Gordon-Larsen, 2001;Dunger *et al.*, 2005) and early maturation may increase overall adult fatness (Kirchengast *et al.*, 1998). In our study we also observed that women experiencing early maturation tended to also have an increased adult body fatness ($p=0.07$).

The close relationship between obesity and age at menarche is supported in a recent intervention study, where age at menarche was delayed as a result of reduction in BMI and body fatness (Chavarro *et al.*, 2005). Thus, an increase in E₂ levels during late childhood and puberty may promote not only the onset of the first menstrual bleeding but also the development of higher amounts of body fat tissue. Moreover, body fat may acts as a secondary hormonal gland, influencing E₂ levels during the whole of adult life (Kirchengast *et al.*, 1998). Thus, our observation that subgroups of premenopausal women, characterized by both early age at menarche and adult overweight and obesity (high BMI, DEXA, WC and WHR), experience higher E₂ levels throughout their menstrual cycle is biologically plausible. Furthermore, energy expenditure may reduce serum E₂ levels (Bernstein *et al.*, 1987;Kumar *et al.*, 2005) and biologically active free E₂ throughout a menstrual cycle (Jasienska *et al.*, 2006), supporting the hypothesis that excess weight may contribute to increased estradiol levels among premenopausal women. Of importance, is also the observation that even among

our women, that were relatively thin, we observed an increase in the level of E₂ with increasing relative body weight (BMI, WC, WHR, DEXA total fat).

It should be noted that, by stratifying by parity, we observed the strongest interrelationship of age at menarche, BMI, WC and E₂ levels observed throughout a menstrual cycle for non-parous women (data not shown). However, no significant interactions were found and due to small numbers in each group these results need to be interpreted with caution. Furthermore, in some studies parous women were observed to have higher premenopausal E₂ levels than non-parous women (Bernstein, 2002; Kaaks *et al.*, 2005) in contrast to others (Verkasalo *et al.*, 2001), but these studies have not been stratified by age at menarche.

To our knowledge, no studies to date have examined if the subgroup characterized by early age at menarche and excessive body weight throughout premenopausal years are at high risk for breast cancer. Furthermore, our results may seem inconsistent with studies that have found a reduced risk of premenopausal breast cancer among women who were obese as young adults (IARC Handbook of Cancer Prevention, 2002; Ballard-Barbash *et al.*, 2006; Ursin *et al.*, 1995). Nevertheless, several studies support our results indirectly; women with central obesity had increased risk of pre- and postmenopausal breast cancer (Wu *et al.*, 2006), newly diagnosed cases of premenopausal breast cancer had significantly higher serum total E₂ levels and higher WHRs than controls (Kumar *et al.*, 2005) and women with follicular total and free E₂ levels in the top 25% had a doubling of breast cancer risk (Eliassen *et al.*, 2006). Furthermore, premenopausal overweight women who developed breast cancer had somewhat higher prediagnostic blood levels of E₂ than those who did not develop breast cancer, which supports the importance of the levels of E₂ among premenopausal women (Travis and Key, 2003). However, many prior studies have not aligned estrogen assays to the same time during

the menstrual cycle making it difficult to compare results across studies (Kaaks *et al.*, 2005; Bernstein, 2002).

Our observations of a higher E₂ level among women who have both an early age at menarche and adult obesity support studies demonstrating a modest increase in breast cancer risk associated with younger age at menarche (Bernstein, 2002; Kelsey *et al.*, 1993). To our knowledge these studies have not stratified by premenopausal body composition. The observed association between age at menarche and breast cancer risk is often explained due to early establishment of regular cycles and longer time between age at menarche and first full-term pregnancy, and hence the overall higher total cumulative dose of estrogen throughout life. Furthermore, age at menarche is a good marker for determining total duration of endogenous estrogen exposure (Hagemans *et al.*, 2004). Any factor that modifies menstrual cycle patterns and reduces the levels of E₂ or the frequency of ovulation may reduce a woman's lifetime risk of developing breast cancer (Bernstein *et al.*, 1994). The observation that obesity in premenopausal women may lower this risk of breast cancer, has been hypothesized to be due to higher frequency of longer anovulatory cycles among obese women (IARC Handbook of Cancer Prevention, 2002; Ballard-Barbash *et al.*, 2006).

Our study has several strengths, including daily saliva sampling for estimation of daily 17- β -estradiol concentrations through an entire menstrual cycle. We used well-developed and validated methods and assays to characterize the women's exposure to free, biologically active E₂ and performed comparisons of levels by aligned cycle days (Lipson and Ellison, 1996). This recommended approach, of examining all women at the same time during the menstrual cycle, is rarely achieved because of its logistic complexity (Kaaks *et al.*, 2005; Bernstein, 2002). Additionally, one trained nurse traced all the participants throughout the study, and all were met in the same clinical research department at a university hospital. This standardization enhanced the quality of our data and allowed us to sample all clinical

variables within the same narrow frame of the cycle for each participant, using uniform procedures. To limit any potential influence of season, women did not participate during months with no daylight (December and January).

In order to study if different anthropometric measurements in particular were associated with levels of E₂, several measurements were included. Of notice, is the observation that even if DEXA % total fat is looked upon as a valid and objective fat measurement method, BMI, WC and WHR gave additional information. One explanation may be that our DEXA measurement does not allow an index of central obesity, as the truncal DEXA fat is measuring fat from the hips to the shoulders. Neither DEXA total fat or DEXA truncal fat, represent central adiposity in particular, in the same matter as WC and WHR.

The present population is very homogenous which may strengthen the interpretation in smaller groups, but it also underlines the need for further studies among other ethnic groups. Additionally, in order to perform stratification by age at menarche and body composition, certain subgroups were small.

Age at menarche was collected retrospectively and misclassification is possible, although several studies have found age at menarche to be recalled with high reliability (Must *et al.*, 2002). In our study we used both interview and a life calendar to improve the accuracy of recall and age at menarche is in accordance with comparable observations in a large prospective study from Norway (Lien *et al.*, 2006).

Conclusion

Reducing the lifetime levels of estrogen has been proposed as the most important step in lowering the risk of breast cancer in women. Our results suggest that women who experience early age at menarche and adult excess weight may experience higher levels of E₂ throughout

a menstrual cycle. These findings underline the necessity to further study variation in biologically active estradiol by subgroups of women, in order to elucidate the associations between lifestyle factors, characteristics of women and breast cancer risk.

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Figure legend:

Figure 1. Age adjusted salivary 17- β -estradiol concentrations by cycle day in women categorized by anthropometric measurements [Body mass index (BMI) (A), total tissue fat (DEXA) (B), waist circumference (WC) (C), and waist to hip ratio (WHR) (D)] and age at menarche over and below 12 years. Linear mixed models for repeated measurement.

- , Group I: Menarche \leq 12 years and anthropometric measurement under 75 percentile
- , Group II: Menarche \leq 12 years and anthropometric measurement over 75 percentile
- , Group III: Menarche $>$ 12 years and anthropometric measurement under 75 percentile
- , Group IV: Menarche $>$ 12 and anthropometric measurement over 75 percentile

A Groups of BMI and age at menarche

- Group I: Menarche \leq 12 yrs and BMI $<$ 26.5 kg/m², n= 34
- Group II: Menarche \leq 12 yrs and BMI \geq 26.6 kg/m², n=15
- Group III: Menarche $>$ 12 yrs and BMI $<$ 26.5 kg/m², n=119
- Group IV: Menarche $>$ 12 yrs and BMI \geq 26.6 kg/m², n=36

B Groups of DEXA and age at menarche

- Group I: Menarche \leq 12 yrs and DEXA $<$ 40 %, n= 34
- Group II: Menarche \leq 12 yrs and DEXA \geq 40 %, n=15
- Group III: Menarche $>$ 12 yrs and DEXA $<$ 40 %, n=119
- Group IV: Menarche $>$ 12 yrs and DEXA \geq 40 %, n=36

C Groups of WC and age at menarche

- Group I: Menarche \leq 12 yrs and WC $<$ 84 cm, n=32
- Group II: Menarche \leq 12 yrs and WC \geq 84 cm, n= 17
- Group III: Menarche $>$ 12 yrs and WC $<$ 84 cm, n=116
- Group IV: Menarche $>$ 12 yrs and WC \geq 84 cm, n=39

D Groups of WHR and age at menarche

- Group I: Menarche \leq 12 yrs and WHR $<$ 0.8, n= 32
- Group II: Menarche \leq 12 yrs and WHR \geq 0.8, n=17
- Group III: Menarche $>$ 12 yrs and WHR $<$ 0.8, n=118
- Group IV: Menarche $>$ 12 yrs and WHR \geq 0.8, n=37

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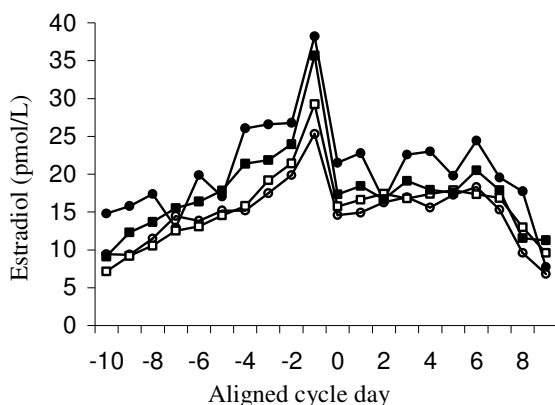
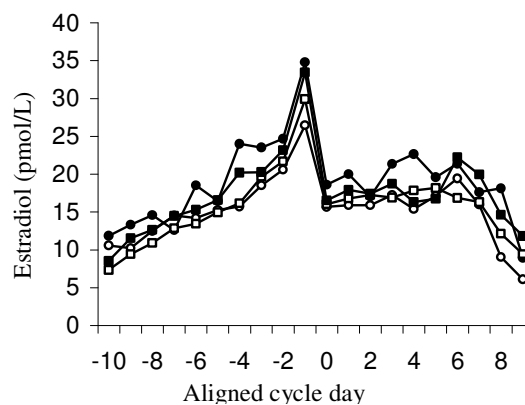
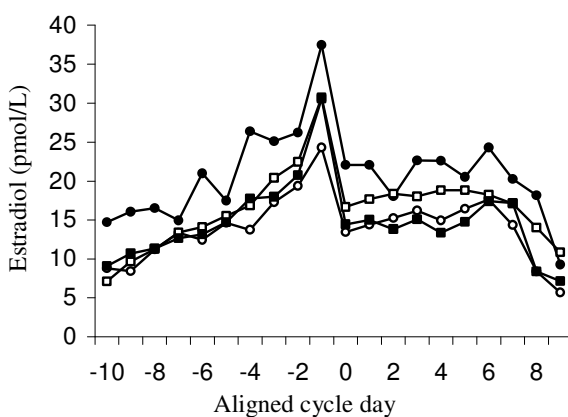
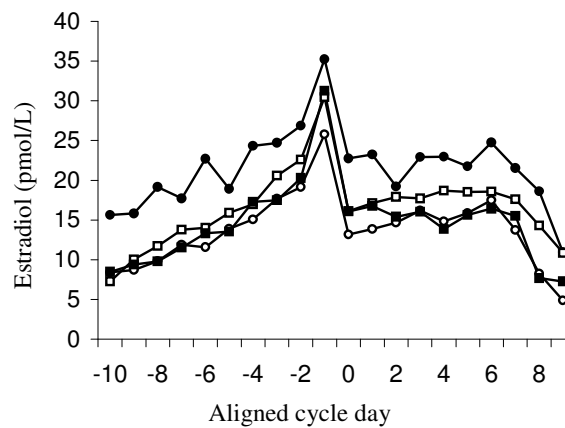
A Groups of BMI and age at menarche**B** Groups of DEXA and age at menarche**C** Groups of WC and age at menarche**D** Groups of WHR and age at menarche

Table I Characteristics of the study population: means, (SDs)* or proportions
The Norwegian EBBA study ($n = 204^{\dagger}$).

| | Menarche ≤ 12 years ($n = 49$) | Menarche > 12–14 years ($n = 100$) | Menarche ≥ 14 years ($n = 55$) | p value ‡ |
|--|---|--|---|-------------------------|
| Age, years | 30.9 (2.8) | 30.4 (2.9) | 31.2 (3.5) | 0.30 |
| Years of schooling | 16.0 (3.1) | 16.1 (3.0) | 16.0 (3.1) | 0.94 |
| Ethnic minority (Sami %) | 12.2 | 6.0 | 7.3 | 0.41 |
| Body composition | | | | |
| Height (cm) | 164.7 (5.3) | 167.4 (7.0) | 167.9 (6.3) | 0.03 |
| BMI (kg/m ²) | 25.3 (4.7) | 24.3 (3.2) | 23.8 (3.7) | 0.11 |
| Waist circumference (WC) (cm) | 81.1 (12.1) | 79.9 (8.9) | 77.5 (8.9) | 0.15 |
| Waist to hip ratio (WHR) | 0.78 (0.07) | 0.77 (0.06) | 0.76 (0.06) | 0.20 |
| Total tissue fat (%) | 35.8 (7.8) | 34.3 (7.5) | 32.3 (7.4) | 0.07 |
| Total cholesterol (mmol/L) | 4.3 (0.7) | 4.5 (0.8) | 4.5 (0.7) | 0.48 |
| HDL cholesterol (mmol/L) | 1.5 (0.3) | 1.6 (0.3) | 1.6 (0.3) | 0.35 |
| LDL/HDL ratio | 1.8 (0.7) | 1.8 (0.7) | 1.7 (0.6) | 0.85 |
| Saliva hormone concentrations (pmol/L) | | | | |
| Overall average 17 β estradiol | 17.8 (9.3) | 17.7 (8.7) | 18.4 (8.5) | 0.90 |
| Serum hormone concentration § (nmol/L) | | | | |
| Serum estradiol | 0.16 (0.07) | 0.14 (0.06) | 0.15 (0.06) | 0.49 |
| Menstrual and reproductive characteristics | | | | |
| Women with children (%) | 49.0 | 47.0 | 49.1 | 0.96 |
| Age at 1st birth(years) $^{\parallel}$ | 24.0 (2.8) | 24.7 (3.8) | 24.6 (4.7) | 0.79 |
| Cycle length (days) | 28.1 (2.9) | 28.1 (3.1) | 28.6 (3.5) | 0.63 |
| Previous use of hormonal contraceptives (%) | 79.6 | 84.0 | 87.0 | 0.59 |
| Energy intake (kJ/day) | 7895 (2033) | 8197 (1897) | 8079 (1801) | 0.66 |
| Leisure time (MET h/week) | 49.8 (34.6) | 52.0 (33.7) | 55.5 (41.5) | 0.72 |
| Alcohol units per week among users ($n = 190$) | 3.0 (4.0) | 2.8 (3.1) | 3.8 (3.5) | 0.20 |
| Current smokers (%) | 22.5 | 23.0 | 20.0 | 0.91 |

*SDs = standard deviation.

† Numbers may vary due to missing information.

‡ One way analysis of variance or χ^2 test.

§ Blood sampling first visit (days 1–5).

$^{\parallel}$ For those who have children: $n = 98$, in each group: 24–47–27.

Table II Estimated changes* in salivary 17- β -estradiol concentration (pmol/L) by 1 standard deviation (SD) increase in explanatory variables in total menstrual cycle (n=204)[†], follicular and luteal phase (n=190)[‡]

| Variables [‡] | Mean (SD) | Mean E ₂ change (n=204) | | | | Mean E ₂ change-follicular index (n=190) [§] | | | | Mean E ₂ change- luteal index (n=190) [§] | | | |
|----------------------------|-------------|------------------------------------|---------|--|---------|--|---------|--|---------|---|---------|--|---------|
| | | Age adjusted effect (95% CI) | p-value | Multivariate adjusted effect (95 % CI) | p-value | Age adjusted effect (95% CI) | p-value | Multivariate adjusted effect (95 % CI) | p-value | Age adjusted effect (95% CI) | p-value | Multivariate adjusted effect (95 % CI) | p-value |
| Age (years) | 30.7 (3.1) | -0.8 (-2.0,0.4) | 0.20 | -1.0 (-2.2, 0.2) | 0.12 | -0.8 (-2.1, 0.5) | 0.22 | -0.9 (-2.2, 0.4) | 0.18 | -1.3 (-2.7, 0.1) | 0.08 | -1.3 (-2.7, 0.1) | 0.07 |
| Menarche (years) | 13.1 (1.4) | -0.3 (-1.5, 1.0) | 0.67 | 0.1 (-1.2, 1.4) | 0.90 | -0.4 (-1.7, 0.9) | 0.53 | -0.2 (-1.5, 1.1) | 0.75 | -0.3 (-1.7, 1.1) | 0.64 | 0.2 (-1.3, 1.6) | 0.83 |
| BMI** (kg/m ²) | 24.4 (3.8) | 2.2 (1.0, 3.4) | 0.001 | 2.2 (1.0, 3.4) | <.001 | 2.4 (1.2, 3.7) | 0.0001 | 2.5 (1.3, 3.8) | <.0001 | 1.7 (0.4, 3.1) | 0.01 | 1.7 (0.3, 3.0) | 0.01 |
| WC** (cm) | 79.5 (9.8) | 1.6 (0.4, 2.8) | 0.01 | 1.6 (0.4, 2.8) | 0.01 | 1.7 (0.4, 2.9) | 0.01 | 1.7 (0.4,2.9) | <.01 | 1.2 (-0.2, 2.5) | 0.09 | 1.1 (-0.2, 2.5) | 0.10 |
| WHR** | 0.77 (0.06) | 1.4 (0.2, 2.6) | 0.02 | 1.1 (-0.1, 2.3) | 0.07 | 1.4 (0.2, 2.7) | 0.03 | 1.3 (-0.0,2.5) | 0.06 | 1.2 (-0.1, 2.6) | 0.08 | 0.9 (-0.5, 2.3) | 0.20 |
| DEXA** (%) | 34.2 (7.6) | 1.4 (0.2, 2.6) | 0.02 | 1.3 (0.1, 2.5) | 0.04 | 1.4 (0.1, 2.7) | 0.03 | 1.4 (0.1, 2.7) | 0.03 | 1.0 (-0.3, 2.4) | 0.14 | 0.8 (-0.6,2.2) | 0.28 |

* Linear regression analyses. Regression coefficient and 95% confidence interval (CI).

† Number may vary due to missing information

‡ Age at entry and measurements at days 1–5 after onset of the menstrual cycle.

§ Includes women with aligned cycles only.

|| Adjusted for age, number of cigarettes, leisure time physical activity and birth weight.

** BMI= Body mass index; WC= Waist circumference; WHR= Waist to hip ratio; DEXA= Total tissue fat (%)

Table III Estimated changes* in salivary overall average 17- β -estradiol concentrations (pmol/L) with 95% confidence interval by 1 standard deviation (SD) increase in explanatory variable[‡] in groups of menarche ($n=204$)[†]

| Variables [‡] | Menarche ≤12 (n = 49) | | | | | Menarche 12-14 (n = 100) | | | | | Menarche ≥14 (n = 55) | | | | | p inter- action |
|----------------------------|--------------------------|------------------------------------|-------------|---|-------------|------------------------------------|-------------|---|-------------|------------------------------------|--------------------------|---|-------------|------|--|--------------------|
| | Mean (SD) | Age adjusted effect (95% CI) | p- value | Multivariate adjusted effect [¶] (95 % CI) | p- value | Age adjusted effect (95% CI) | p- value | Multivariate adjusted effect [¶] (95 % CI) | p- value | Age adjusted effect (95% CI) | p- value | Multivariate adjusted effect [¶] (95 % CI) | p- value | | | |
| Mean estradiol (pmol/L) | | | | 17.8 | | | | 17.7 | | | | | 18.4 | | | |
| BMI** (kg/m ²) | 24.4 (3.8) | 3.3 (1.2, 5.3) | 0.002 | 3.4 (1.4,5.5) | 0.001 | 1.4 (-0.6, 3.5) | 0.2 | 1.4 (-0.7, 3.5) | 0.2 | 1.9 (-0.5, 4.3) | 0.1 | 1.3 (-0.9, 3.6) | 0.2 | 0.33 | | |
| WC ** (cm) | 79.5 (9.8) | 3.6 (1.7, 5.6) | <.001 | 3.7 (1.8, 5.7) | <.001 | 0.2 (-1.8, 2.1) | 0.9 | 0.2 (-1.8,2.2) | 0.8 | 1.1 (-1.5, 3.7) | 0.4 | 0.6 (-1.8,3.1) | 0.6 | 0.07 | | |
| WHR** | 0.77(0.06) | 4.1 (1.9, 6.3) | <.001 | 4.3 (2.0, 6.5) | <.001 | 0.4 (-1.3, 2.2) | 0.7 | 0.3 (-1.5, 2.1) | 0.7 | 0.4 (-2.2, 3.1) | 0.7 | -0.6 (-3.1,2.0) | 0.7 | 0.03 | | |
| DEXA** (%) | 34.2 (7.6) | 2.8 (0.2, 5.4) | 0.04 | 2.9 (0.2,5.5) | 0.03 | 1.3 (-0.4, 3.0) | 0.1 | 1.0 (-0.9,2.8) | 0.3 | 0.7 (-1.7, 3.1) | 0.6 | -0.01(-2.3,2.3) | 1.0 | 0.22 | | |

* Linear regression analysis. Regression coefficient and 95% confidence interval (CI)

[†] Number may vary due to missing information.

[‡] Measurements at days 1–5 after onset of the menstrual cycle.

^{||} Adjusted for age, number of cigarettes, leisure time physical activity and birth weight.

** BMI= Body mass index; WC= Waist circumference; WHR= Waist to hip ratio; DEXA= Total tissue fat (%)